GENETIC DIFFERENTIATION OF WHITE RHINOCEROS SUBSPECIES: DIAGNOSTIC DIFFERENCES IN MITOCHONDRIAL DNA AND SERUM PROTEINS

M. George, Jr.[1], L.G. Chemnick[2], D. Cisova[3], E. Gabrisova[3], A. Stratil[3], and O.A. Ryder[2]
[1]Howard University, Department of Biochemistry and Molecular Biology, College of Medicine, Washington, D.C. 20059 USA.
[2]Zoological Society of San Diego, Center for Reproduction of Endangered Species P.O. Box 551, San Diego, CA 92112 USA.
[3]Institute of Animal Physiology and Genetics, Czechoslovak Academy of Sciences Libechov 27721, Czechoslovakia

ABSTRACT

Mitochondrial DNA and serum proteins of white rhinos (*Ceratotherium simum*) have been analyzed for evidence of subspecies differences. The northern white rhinoceros (*C. s. cottoni*) and the southern white rhinoceros (*C. s. simum*) can be distinguished by recognition sites for ten different restriction endonucleases and by the presence or absence of a serum esterase, ES3. Results derived from the larger sample set utilized in these studies reinforce the phylogenetic distinctiveness of the two geographic forms of white rhinoceros, although estimates of their divergence based on mtDNA analyses have decreased from 4% to 1.4%. New examples of biochemical and molecular genetic variation in African rhinos have been identified. However, the overall genetic variation in African rhinos remains low in comparison to other species of rhinoceros examined and other mammals.

INTRODUCTION

The conservation strategies for African rhinos is increasingly focused on the creation and management of protected sanctuaries to hold rhino populations that can be allowed to grow at optimal rates. As soon as is feasible, rhinos from sanctuaries can be utilized to re-stock former habitat. However, it is not yet clear whether maintenance of genetically small populations of rhinos in protected sanctuaries will constitute a short term or long term conservation action. Rhino sanctuaries may be stocked either by a remnant resident population or by animals translocated from outside areas. Inevitably, the question arises as to what extent rhino populations existing in differing habitat types represent ecotypes of rhinos or whether different geographical populations correspond to subspecies whose variation may be significant in an evolutionary sense. This concern can arise at the onset of a sanctuary-based conservation program if translocation of animals from different geographic or bio-regions is considered. A similar concern can also arise in the future when individuals from sanctuaries are considered for translocation to bolster depleted populations or start new ones in other habitats or regions.

As a consequence of these concerns, the importance of understanding historical zoogeography of rhino populations, including genetic aspects of population variation and divergence has received increasing attention, even as rhino populations decline and the opportunity to undertake such studies diminishes. For these reasons, the extent of genetic variation among the African rhinoceroses is now the subject of detailed investigation Mitochondrial DNA (mtDNA) studies of black rhinos, *Diceros bicornis*, showed very little variation among different subspecies (0.18-0.24%) (Ashley *et al.*, 1990). Allozyme variation studies (Merenlender *et al.*, 1989) showed little intraspecific variation among African and Indian rhinoceroses, although samples sizes were small. The level of polymorphism ranged between 0.0-0.1% and mean heterozygosity ranged between 0.0-0.02%. A preliminary study focusing on the utility of using mtDNA as a means of discriminating between the subspecies of white rhino, *Ceratotherium simum*, reported that the northern subspecies (*C. s. cottoni*) differed from the southern subspecies (*C. s. simum*) by an estimated 4% in their mtDNA nucleotide sequences (George *et al.*, 1983). This study

suffered from small sample sizes as only one northern white rhino, one southern white rhino and one black rhino were examined.

We have attempted to assemble a larger sample set for comparison of within and between population genetic differentiation in African rhinoceroses, with emphasis on examination of the two geographical subspecies of the white rhinoceros. This study examines mtDNA restriction fragment length variation in six unrelated northern white rhinos, six southern white rhinos and six black rhinos (belonging to two subspecies). The number of restriction enzymes utilized in these analyses has been increased from 21 to 33. Variation in nuclear loci encoding proteins present in serum has also been examined. Polymorphism in serum proteins of *Ceratotherium simum* has been observed and a serum esterase is identified that is diagnostic for the two white rhino subspecies.

MATERIALS AND METHODS

Table 1 lists the types and sources of tissues used for mitochondrial DNA (mtDNA) analysis. For serum protein studies, blood samples of Southern white rhinoceros *(Ceratotherium simum simum)* (7 animals) were collected from the herd at San Diego Wild Animal Park. Serum samples were kept at -20°C. Serum samples of Northern white rhinoceros *(C. s. cottoni)* from Zoo Dvur Kralove were the same as in Stratil *et al.* (1990), and one sample was from San Diego Zoo (studbook number 1413, Lucy). Two serum samples of east African black rhinoceros, *Diceros bicornis michaeli*, (one from San Diego and one from Dvur Kralove) were also used.

One-dimensional polyacrylamide gel electrophoresis (1D PAGE) and two-dimensional agarose gel (pH 5.4) - polyacrylamide gel (pH 9.0) electrophoresis (2D agarose - PAGE) were performed as described by Juneja and Gahne (1987) and Stratil *et al.* (1990). The gels were stained with Coomassie Blue G-250, or blotted to nitrocellulose sheets. Immunoblotting (to detect A1BG and GC) was carried out as described by Stratil *et al.* (1990). Esterase was stained with a-naphthylacetate (100 ml 0.08 M Tris-citrate buffer, pH 6.5, 100 mg α-naphthylacetate and 100 mg Fast Blue BB salt).

Mitochondrial DNA was isolated and purified from organ tissue(s) of northern and southern white rhinos and a black rhino according to procedures described by George (1982). High molecular weight genomic DNAs were prepared by methods described by Alexander *et al.* 1987) and Robbins *et al.* (1979).

The genomic and mtDNAs were subjected to restriction enzyme digestion with the 33 enzymes listed in Table 2. The digests were performed as per instructions of the various manufacturers (Life Technologies-Bethesda Research Labs; New England Biolabs; International Biotechnologies, Inc.). The DNAs were digested at the recommended temperature for a minimum of 2 hours to a maximum of 18 hours. Following digestion, the DNAs were electrophoresed in agarose gels. Genomic DNAs were electrophoresed on either 0.8% or 1.0% agarose gels for Southern blot experiments. Digested mtDNAs were usually separated on 1.2% agarose gels after end labeling the DNA with α-^{32}P nucleotides and subjected to autoradiography (Brown, 1980).

Southern blot hybridization experiments were done according to the procedures of Southern (1975), Maniatis *et al.* (1982), and Rigaud *et al.* (1987). Mitochondrial DNAs of northern or southern white rhinos were labeled via the random prime labeling method of Feinberg and Vogelstein (1983; 1984). The random primed labeled mtDNAs were used as probes to detect the mtDNA content of the genomic DNAs. The hybridized blots were subjected to autoradiography for 1-12 days at -70° C. DNA markers (λ bacteriophage DNA digested with *Hind III* and SP6 bacteriophage DNA digested with *Kpn I* and *Hind III*) were also labeled via random priming and run on gels containing the genomic DNAs. Some gels also contained purified digested mtDNA.

The Southern blot gels of the rhino DNAs were analyzed by the methods of Nei and Li (1979); the Mac PAUP computer program (Phylogenetic Analysis Using Parsimony for the MacIntosh computer by David Swofford, University of Illinois); and the DVAL computer program obtained from L. Gentzbittel, the University Blaise Pascal, Biologie Moleculaire Vegetale, Paris.

RESULTS

Mitochondrial DNA Comparisons

Based on comparison of 129 restriction fragments in the northern white rhino and 128 restriction fragments in the southern white rhino, 108 fragments were held in common by the two groups, corresponding to a F-value of 0.840 (Nei and Li, 1979) and an estimated nucleotide sequence divergence of 1.4%. The lower estimate for nucleotide sequence divergence arose as a result of increasing the number of restriction enzymes utilized in the analysis rather than through the inclusion of a larger number of individuals in the study.

Ten restriction endonucleases (*Pvu II, Ava I, Acc I, Hind III, Hae III, Hinc II, Msp I, Dra I, Hpa I* and *Taq I*) produced digestion patterns that distinguished northern and southern white rhinos. Seven of these enzymes (*Pvu II, Ava I, Acc I, Hinc II, Hpa I, Hind III and Hae III*) found to be diagnostic for population origin on the minimal sample set of George, *et al.* (1983) still provided distinctive differences in cleavage patterns with the larger sample set. In addition to surveying a larger number of white rhinos, an additional 12 restriction endonucleases were tested and three retested on the larger sample set. Three of these proved to be diagnostic. Increasing the number of restriction enzymes about doubled the number of fragments used for comparison in the earlier study (George *et al.*, 1983). Results with the larger sample set identify two distinct population haplotypes corresponding to the geographical subspecies.

Both northern white and southern white rhinos differed from black rhinos by approximately 4.5% nucleotide sequence divergence. Intraspecific variation in black rhino mtDNA restriction patterns was produced by *Acc I, Bcl I, Hinc II, Taq I, BstU I, Stu I, Hinf I and Msp I.*

Restriction endonuclease *Taq I* produced fragment patterns that were diagnostic for the examined subspecies of both African rhino species (Figure 1). This enzyme had previously been reported to distinguish *D. b. michaeli* and *D. b. minor* by Ashley, *et al.* (1990). This enzyme also identified a polymorphism within *C s. cottoni*, (Figure 1) which represents the first description of genetic variability at the DNA level in this taxon. We have not observed similar mtDNA restriction cleavage site polymorphisms in *C. s. simum.*

Serum protein comparisons

A comparison of serum proteins of *C. s. simum* and *C. s. cottoni* in 1D PAGE, 2D agarose - PAGE (Figure 2) and immunoblotting revealed no differences between the two subspecies in electrophoretic migration of most of the studied proteins (i.e. albumin - ALB; transferrin - TF; GC protein - GC; postalbumin - PSA; haptoglobin - HP; inhibitors of chymotrypsin - AC; inhibitors of trypsin - AT1 and AT2, and inhibitors of both trypsin and chymotrypsin - ATC1 and ATC2). Some of the proteins that exhibited polymorphism in *C. s. cottoni* (Stratil *et al.*, 1990) were monomorphic in *C. s. simum* - AC, GC, HP and ATC1 (Table 3).

Two proteins, A1BG and ES3, were of great interest in *C. s. simum.* While in *C. s. cottoni* A1BG was monomorphic (Stratil *et al.*, 1990), in *C. s. simum* it exhibited polymorphism. Three phenotypes were observed - F, FS and S, which are apparently under genetic control by two codominant alleles, $A1BG^F$ and $A1BG^S$. It is interesting to note that in the first dimension agarose gel electrophoresis (pH 5.4) variant F migrates slower than variant S, while in the second dimension PAGE (pH 9.0) F is faster than S. This system can easily be studied also in 1D PAGE. It is interesting to note that polymorphism of A1BG was observed also in two samples of *D. bicornis michaeli.* One animal was A1BG S and one A1BG FS (Figure 2).

Staining of serum samples of *C. s. simum* after 1D PAGE for esterase revealed three zones - ES1, ES2 and ES3 (Figure 3). One sample (S7) appeared as a heterozygote in ES1 and ES2.

Of great interest was the presence of ES3 in all *C. s. simum* studied. In an earlier study on serum proteins of *C. s. cottoni* all but one animal lacked ES3 (Stratil *et al.*, 1990). This esterase zone was present only in a hybrid between *C .s. cottoni* and *C. s. simum*.

DISCUSSION

Two previous studies of biochemical and molecular genetic variation in the African white rhinoceros, *Ceratotherium simum*, produced conflicting findings. A comparison of restriction endonuclease cleavage patterns in the mtDNA of one northern white rhino, one southern white rhino and one black rhino resulted in an estimate of 7% nucleotide sequence difference between the black rhinoceros (*Diceros bicornis*) and the white rhinoceros (George *et al.*, 1983). The northern and southern subspecies of the white rhino (*C. s. cottoni* and *C. s. simum*, respectively) displayed different cleavage patterns with a number of restriction enzymes resulting in an estimated nucleotide sequence divergence of approximately 4%, or approximately 57% of the difference between the two genera of African rhinos.

In a study of blood and tissue allozymes the only differences detected in 7 northern white and 23 southern white rhinos were in a serum protein, AB-3, (presumed to be transferrin) and PGM-2 (Merenlender *et al.*, 1989), The estimated Nei distance was a trivial 0.005. A previous study of serum protein variation in white rhinos included only six individuals of the northern subspecies and one subspecies hybrid individual. The comparative analysis by gel electrophoresis of individual white rhinos of both subspecies identified that ES3 was present in 23 individuals of the southern subspecies and the one subspecies hybrid. Serum ES3 was not observed in northern white rhinos. In addition to the presence of ES3, southern white rhinos displayed polymorphism in α-glycoprotein (A1BG) and variation in ES1 and ES2. However, in a previous study (Stratil *et al.*, 1990), several proteins found to be polymorphic in the northern subspecies were monomorphic in the southern white rhinos analyzed in this study.

Based on the samples utilized here it is tempting to conclude that the two subspecies can be differentiated on the basis of the presence or absence of ES3; *C. s. simum* possesses ES3, while *C. s. cottoni* lacks this esterase. In the one subspecies hybrid examined, ES3 was detected, suggesting codominant or dominant expression of ES3. If this conclusion is correct, ES3 alone could be used to distinguish between the two subspecies.

For this study a larger number of samples for mitochondrial DNA analysis was assembled. This necessitated that restriction fragments be detected by Southern blot hybridization rather than by direct analysis of purified mtDNAs. Digestion fragments were detected on Southern blots of genomic DNA hybridized with purified labeled mtDNA that were not observed when purified mtDNA was digested, suggesting that the nuclear genome was the source of these extra bands. However, the nature of these additional fragments have yet to be explained. The unique fragments may be of phylogenetic importance once characterized. At this time we conclude that the extra fragments may represent mtDNA fragments which are harbored in the chromosomes of the nucleus, may represent mtDNA duplications or mtDNA heteroplasmy.

The mtDNA of six northern white rhinos was examined in this study. These individuals were not known to be related. An increase of sample size from one to six may not, at first, seem an adequate improvement in sample size. However, there can only be one additional unrelated mtDNA clone in the entire captive population. Furthermore, as the wild population decreased to a low number of approximately 19 individuals (including offspring), it is unlikely that even 10 different unrelated mtDNA clones were present in the surviving wild population. As a rough estimate, the six mtDNA samples utilized in this study may represent at least one-third of the total subspecies mtDNA haplotype diversity.

It should be pointed out that opportunities for obtaining samples for genetic analysis of rhino populations have been limited and captive populations of rhinos have been the greatest source of useful material. This may change in the future as more detailed analysis of rhino population genetics is desired and as new techniques render such studies more feasible as a result of less invasive sampling requirements (Ryder, 1992; Garner and Ryder, 1992).

The six black rhinos examined in this study represent 2 subspecies, East African *D. b. michaeli*, and a more broadly distributed southern subspecies, *D. b. minor*. Intraspecific polymorphism in *D. bicornis* was observed utilizing eight restriction enzymes. Based on our samples, restriction endonuclease *Taq I* (Figure 1) and three other enzymes (*Bcl I, BstU I and Stu I*) produced fragments distinguishing the two subspecies. Thus, mtDNA analysis can conceivably be used for subspecies identification consistent with the findings of Harley and O'Ryan (this volume). However, the relatively small sample size, the lack of inclusion of additional subspecies, and the utilization of fewer restriction enzymes than were employed in the comparison of the northern and southern white rhinos all reflect the fact that the principal focus of this study is characterization of genetic differences between northern and southern white rhinoceroses.

Variation in serum proteins appears to be relatively low in the white rhino. Only 6 variable loci have been elucidated with certainty. Both southern and northern white rhinos have experienced severe population bottlenecks requiring recovery from single populations in their natural habitat. The dynamics of their population decline over the last several centuries may differ significantly from that of the greater one-horned rhinoceros, a species that retains high levels of genetic variability (Dinerstein and McCracken, 1990).

The intrasubspecific variability of mtDNA was low for both the northern and southern white rhino (0.0-0.07% and 0.0-0.04% respectively). The white rhinos were found to differ from the black rhinos by an estimated 4.5% mtDNA nucleotide sequence divergence. This is lower than a previous estimate of approximately 7% nucleotide divergence between *Ceratotherium* and *Diceros* (George *et al.*, 1983). The newer estimate for the *Diceros* - *Ceratotherium* divergence would imply divergence of these taxa from a common ancestor approximately 2 million years before present, assuming a rate of mtDNA divergence based on primate taxa (Brown *et al.*, 1979). Alternatively, the average rate of mtDNA divergence may be slower in rhinos than in primates. Fossil evidence supports a divergence time for *Diceros* - *Ceratotherium* of approximately 8 million years before present (Prothero, this volume). The 1.4% estimated nucleotide sequence divergence for the southern white and northern white rhinos also represents a reduction from the previous estimate of approximately 4%. Thus, estimated mtDNA divergence for both the *Diceros* - *Ceratotherium* and for *C. s. simum* and *C. s. cottoni* have been reduced (by 42% and 65%, respectively).

The lower estimate for mtDNA nucleotide sequence divergence resulted from the comparison of a larger number of restriction products than examined previously, not from decreasing the number of restriction enzymes that produced distinctive cleavage patterns in the two populations. George *et al.* (1983) compared 70 fragments in *C. s. cottoni* and 69 fragments in *C. s. simum* produced by 19 of the 21 restriction endonucleases. In the present study, the number of fragments compared was approximately doubled so that 129 fragments in *C. s. cottoni* and 128 fragments in *C. s. simum* produced by 33 restriction endonucleases were compared.

This study corroborates the evidence for phylogenetic separation of northern and southern white rhinos obtained previously, although the extent of nucleotide sequence divergence is smaller than the previous estimate (George *et al.*, 1983). Recognition sites for ten restriction endonucleases and the activity of a serum esterase (ES3) appear now to be diagnostic characters for the two geographic populations recognized as subspecies.

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TABLE 1. Rhinoceros Samples utilized in this study.[a]

		Tissue(s)	Source [c]
Northern White Rhinos			
C. s. cottoni	Lucy (OR300;KB3731)	Liver, spleen, tissue culture	SDZ
C. s. cottoni	Dinka (10529)	Buffy coat	SDZ
C. s. cottoni	Suni	Buffy coat	DK
C. s. cottoni	Nesari	Buffy coat	DK
C. s. cottoni	Nadi (KB5764)	Buffy coat, tissue culture	DK
C. s. cottoni	Sudan (KB5766)	Buffy coat, tissue culture	DK
C. s. cottoni	Nasi (KB5767) [b]	Buffy coat, tissue culture	DK
Southern White Rhinos			
C. s. simum	Mavula (MO100264)[d]	Buffy coat	WAP
C. s. simum	(OR533)	Placenta	SDZ
C. s. simum	(OR523)	Placenta	SDWAP
C. s. simum	(OR101)	Liver, spleen	SDWAP
C. s. simum	(OR505)	Liver, spleen	SDWAP
C. s. simum	(OR125)[d]	Liver, spleen	SDWAP
C. s. simum	(OR361)	Spleen	SDZ
Black Rhinos			
D. b. minor	Gus (KB6119)	Tissue culture	Los Angeles Zoo (LAZ)
D. b. minor	Mabel	White blood cells	LAZ
D. b. michaeli	Lenny (OR331)	Spleen	SDZ
D. b. michaeli	(OR818)	Liver; heart	SDZ
D. b. michaeli	(OR733)[e]	Placenta	SDZ
D. b. michaeli	(OR656)	Heart; liver; spleen	SDZ
D. b. michaeli	(OR492)[e]	Spleen; liver	SDZ

[a] Names and numbers in parentheses represent identification numbers given these samples at CRES, Zoological Society of San Diego. Numbers preceded by KB refer to tissue culture samples.
[b] "Nasi" is a hybrid cross between a male southern white rhino and a female northern white rhino. "Nasi" also has the same mother as "Suni".
[c] Institution abbreviations: SDZ: San Diego Zoo, SDWAP: San Diego Wild Animal Park , DK: Zoo Dvur Kralove
[d] "Mavula" is the dam of OR125.
[e] OR733 and OR492 have the same mother.

TABLE 2. Restriction Enzymes Used in the Cleavage of Rhino DNAs

1. Acc I*	12. Nsi I	23. Pst I
2. Alu I	13. Pvu II*	24. EcoR I
3. Ava I*	14. Rsa I	25. Kpn I
4. Bcl I	15. Sal I	26. Sac II
5. EcoR V	16. Sma I	27. Xho I
6. Hae III*	17. Stu I	28. BstE II
7. Hinc II*	18. Taq I*	29. BstU I
8. Hind III*	19. Hinf I	30. Sst I
9. Msp I*	20. Hpa II	31. Dra I*
10. Hpa I*	21. BamH I	32. Nci I
11. Mbo I	22. Bgl II	33. Xba I

* Diagnostic differences between *C. s. simum* and *C. s. cottoni* were identified by digestion with these enzymes.

Table 3 Phenotypes in serum protein systems of *C.s. simum* and *C.s. cottoni* from San Diego Zoo

Sample Designation	Number	Sex	Name	Phenotypes							
				AC	GC	ATC2	HP	PSA	ATC1	A1BG	ES
C.s. simum											
S1	13256	M	Damu	F	F	FS	F	-	a	FS	1, 2, 3
S2	13260	F	Macite	F	F	FS	F	+	a	FS	1, 2, 3
S3	13362	M	Stb#40	F	F	S	F	+	a	S	1, 2, 3
S4	13396	M	Mdomo	F	F	S	F	+	a	F	1, 2, 3
S5	13400	F	Kava	F	F	S	F	+	a	F	1, 2, 3
S6	13404	F	Bumper	F	F	FS	F	+	a	FS	1, 2, 3
S7	13499	F	Uzima	F	F	S	F	+	a	FS	1^{F}-1, 2^{F}-2, 3
C.s. cottoni											
C7	1413	F	Lucy	F	F	FS	F	+	a	S	1, 2

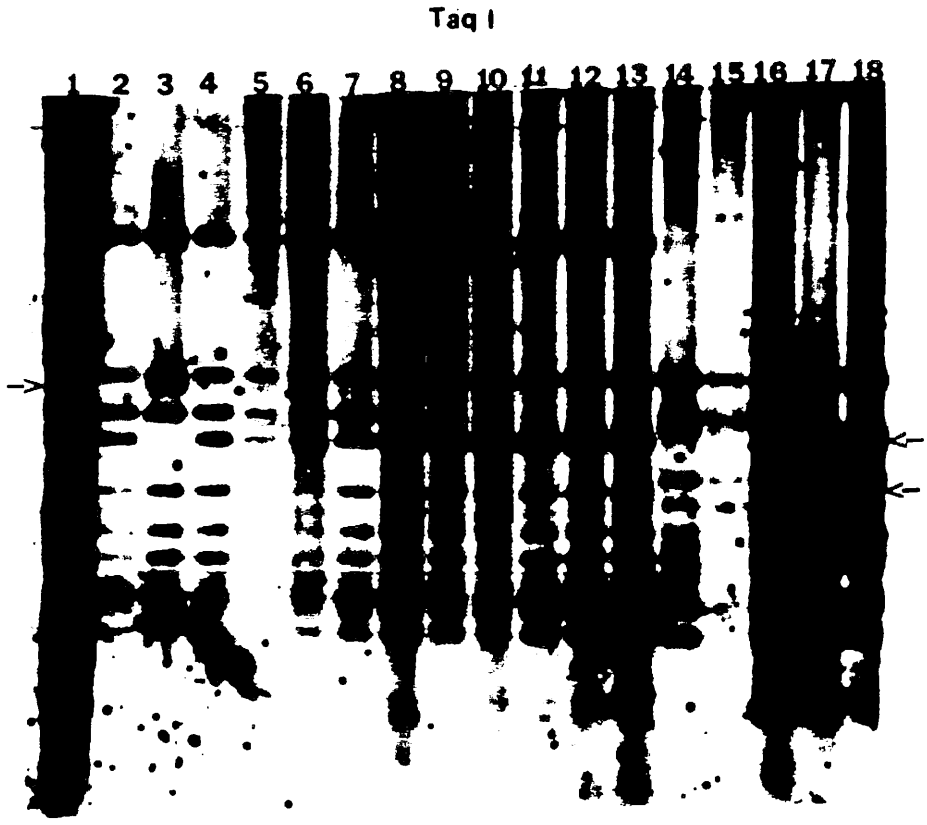


Figure 1.
Southern blot hybridization of Taq I digested DNAs. The marker DNA (Lambda/Hind III) is in lane 1. Lanes 2-7 contain northern white rhino genomic DNAs (Lucy, Nadi, Sudan, Dinka, Nesari and Nasi respectively); lanes 8-13 contain southern white rhino genomic DNAs (OR101, OR125, OR361, OR505, OR523, and OR533, respectively); and lanes 14-18 contain black rhino genomic DNAs ("Gus", "Mabel", OR733, OR818 and OR656, respectively). Labelled, purified southern white rhino mtDNA was used as a probe for these Southern blotted DNAs.

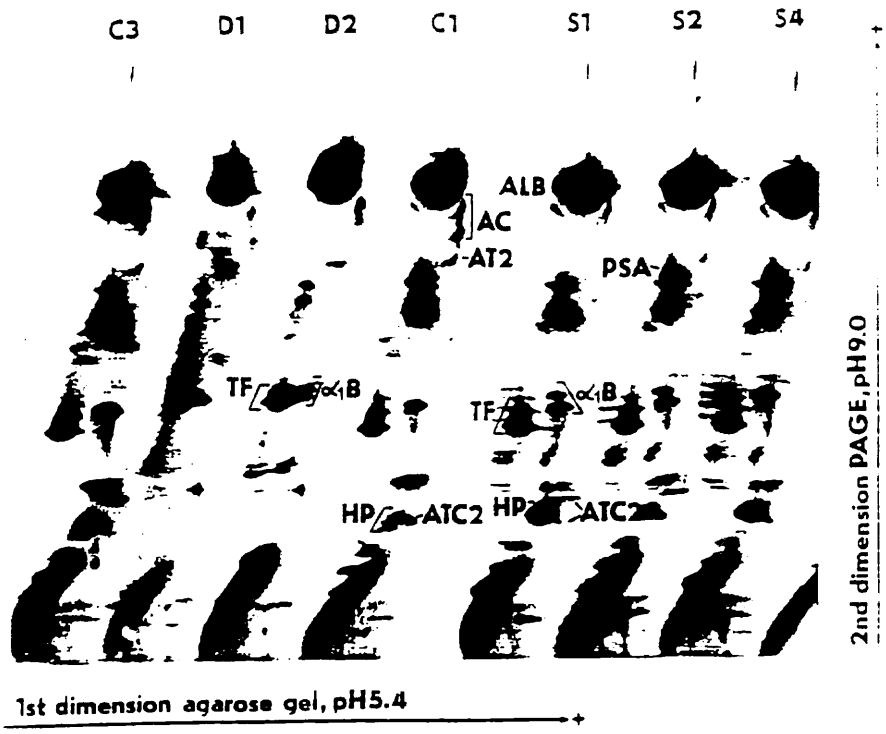
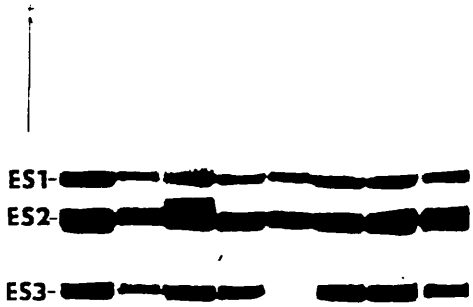


Figure 2
Comparison of serum proteins of *C.s. simum* (S), *C.s. cottoni* (C) and *D. bicornis* (D) in 2D agarose - PAGE. (Coomassie Blue G-250 staining). For sample numbers S1, S2, S4 see Table 1. Samples C1 and C3 are the same as in Stratil et al. (1990). D1 is from Dvur Kralove Zoo, and D2 (No. 12437, Shelbani) from San Diego Zoo. Phenotypes of the studied systems are given in Table 1, and for those of *C.s. cottoni* from Dvur Kralove, see Stratil et al. (1990). *D. bicornis*, sample D1 is A1BG S and D2 is A1BG FS. ALB - albumin; TF - transferrin; a_1B - a_1B glycoprotein (A1BG system); PSA - postalbumin; HP haptoglobin; AC - inhibitor of chymotrypsin; AT2 - inhibitor of trypsin; ATC2 - inhibitor of both trypsin and chymotrypsin. (For details of protein identifications, see Stratil et al., 1990).



S5 S6 S7 C4 C7 S1 S2 S3

Figure 3.
1D PAGE showing serum esterases of *C. s. simum* (S) and *C. s. cottoni* (C). For sample numbers S1, S2, S5, S6, S7 and C7, see Table 3. C4 is Nasi (Dvur Kralove; see Stratil et al., 1990) and she is a hybrid between *C. s. cottoni* and *C. s. simum*.